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GEELNERVIGHEID, EEN VIRUSZIEKTE VAN LUZERNE
(VEIN YELLOWING, A VIRUS DISEASE OF LUCERNE)

DOOR

J. P. H. VAN DER WANT EN L. BOS

INVESTIGATIONS ON WHITE CLOVER MOSAIC VIRUS

DOOR

L. BOS, B. DELEVIĆ AND J. P. H. VAN DER WANT

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GEELNERVIGHEID, EEN VIRUSZIEKTE VAN LUZERNE[1]

With a summary:

Vein yellowing, a virus disease of lucerne

DOOR

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INLEIDING

In het voorjaar van 1950 werden van de Plantenziektenkundige Dienst te Wageningen enige luzerneplanten (*Medicago sativa* L.), die op de bladeren een briljante, gele nerftekening vertoonden, ontvangen, met het verzoek na te gaan of hier van een virusziekte sprake kon zijn. Ondanks herhaalde pogingen om met sap uit de betrokken planten een virus op luzerne, klaversoorten, erwt en tuinboon over te dragen, werden hierbij geen resultaten geboekt.

In hetzelfde jaar nam de eerste schrijver in luzernepercelen op Zuid-Beveland verschijnselen waar overeenkomend met die van de planten, welke van de Plantenziektenkundige Dienst waren ontvangen. In latere jaren werden deze symptomen in verscheidene luzernepercelen waargenomen.

Op grond van in dit artikel te beschrijven kasproeven en waarnemingen in het veld kon worden vastgesteld, dat de onderhavige gele nerftekening op de bladeren van luzerne het gevolg is van infectie der desbetreffende planten met het topvergelingsvirus van de erwt.

SYMPTOMEN

Het verschijnsel is gekenmerkt door een regelmatige, scherp begrensde verkleuring van de nerven en het daaraangrenzende bladmoes (fig. 1). De verkleuring kan variëren van lichtgroen, waarbij de breedte van de verkleurde delen smal is, tot intens geel als de verkleuring zich over een groter oppervlak van de bladschijf uitstrekt. Soms blijft slecht weinig bladmoes met een normale groene kleur over. Deze bladeren wijken doorgaans in vorm en grootte niet af van de bladeren van een gezonde plant. Aangezien het patroon van de nervatuur door de vergeling zo sprekend wordt en zeer regelmatig is, stellen wij voor het verschijnsel „geelnervigheid" te noemen.

Merkwaardig is, dat het ziektebeeld veelal in het voorjaar in luzernevelden kan worden waargenomen, doch dat het doorgaans in de loop van de zomer verdwijnt om in het najaar weer te voorschijn te komen. Ook planten met geelnervigheid, die in de kas groeien, vertonen de tendens in de zomer normaal groene bladeren te vormen. Het is niet zeker, waar dit aan geweten moet worden. Vermoedelijk is de hoeveelheid licht, die de planten ontvangen, bepalend voor het al of niet optreden van de verschijnselen van geelnervigheid (zie ook blz. 76).

[1] Aangenomen voor publikatie 20 februari 1959.
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In juli 1956 werden – naast enige erwteplanten ras Unica – een drietal luzerneplanten, van het in Nederland het meest geteelde ras Du Puits, bezet met verscheidene exemplaren van de erwtebladluis (*Acyrthosiphon pisum* HARRIS), welke geruime tijd hadden gezogen op vergelingszieke erwten, die Ir. N. HUBBELING in een kas op het I.P.O. in observatie had. Twee dagen nadat de luizen op de planten waren geplaatst, werden zij met een insekticideoplossing (TEPP) gedood. De erwteplanten vertoonden na enkele weken symptomen van topvergeling, maar aan de luzerneplanten konden maandenlang geen bijzondere verschijnselen worden waargenomen. In september werd bij één plant een lichtgroene verkleuring van de nervatuur waargenomen. Pas in december 1956 vertoonde deze en een tweede van de drie geïnoculeerde luzerneplanten verschijnselen, die geheel geleken op de hierboven beschreven symptomen van geelnervigheid.

In september 1956 werden virusvrije erwtebladluizen geplaatst op bovengenoemde luzerneplant met zwakke nerfverkleuring. Vervolgens werden zij overgebracht op jonge, gezonde erwteplanten van het ras Unica (vier tot tien luizen per plant). Van de vijf in deze proef gebruikte erwteplanten, waren er na enige weken twee vergelingsziek.

Van een der geelnervige luzerneplanten werden verscheidene stekken genomen, die tot beworteling werden gebracht. Aldus kon het materiaal op eenvoudige wijze voor verder onderzoek in stand worden gehouden.

In mei 1957 werd opnieuw getracht het topvergelingsvirus met erwtebladluizen uit de geelnervige luzernekloon op erwteplanten (ras Unica) over te brengen. Ook thans werd een positief resultaat verkregen: van de 20 planten werden er 11 ziek.

Een derde proef werd genomen in augustus 1957. De erwtebladluizen zogen enige dagen op de geelnervige luzerne en werden vervolgens op vier erwteplanten overgebracht. Deze planten reageerden alle met topvergeling.

Uit deze proeven kan worden geconcludeerd, dat het topvergelingsvirus met behulp van *Acyrthosiphon pisum* van erwt op luzerne kan worden overgebracht en vandaar weer op erwt. Aangezien bij geen van de in de loop der jaren in de kas uit zaad opgekweekte luzerneplanten ooit verschijnselen van geelnervigheid werden waargenomen, behalve bij de met topvergelingsvirus besmette planten, kan worden aangenomen, dat geelnervigheid een gevolg is van infectie met topvergelingsvirus.

Om dit nader aan te tonen werden in het voorjaar van 1957 op Zuid-Beveland en in West-Brabant een zestal monsters luzerneplanten verzameld en op aanwezigheid van het topvergelingsvirus onderzocht. Vijf der betrokken monsters vertoonden geelnervigheid; het zesde had deze verschijnselen niet. Op alle monsters werden virusvrije erwtebladluizen gezet en deze werden enige dagen later overgebracht op gezonde Unica-erwten. Aldus kon uit vier van de vijf monsters met geelnervigheid het topvergelingsvirus worden geïsoleerd. Uit het monster luzerne zonder geelnervigheid brachten de bladluizen geen topvergelingsvirus over.

Hierbij moet worden opgemerkt, dat deze proeven werden bemoeilijkt, doordat de gebruikte stam van de erwtebladluis in de kas op luzerne vaak slechts met moeite tot zuigen kon worden gebracht.

Verder verkregen wij, ook bij het gebruik van bladluizen, die geruime tijd (48 uur of langer) op een topvergelingszieke erwteplant hadden gezogen, bij proefplanten die met 5 tot 10 bladluizen waren bezet, slechts vrij zelden 100 % infectie.

VERSPREIDING VAN GEELNERVIGHEID IN HET VELD

Om te onderzoeken in welke mate het verschijnsel van de geelnervigheid zich in het veld uitbreidt, werd te Wageningen een veldproef genomen (fig. 2). In 1957 waren in drie dubbele rijen luzerne (in fig. 2 gearceerd) planten van de geelnervige luzernekloon geplant en in de loop van het jaar verbreidde de geelnervigheid zich in belangrijke mate van hieruit over de drie rijen. In het voorjaar van 1958 werd de oppervlakte tussen de rijen met luzerne volgezaaid. Bovendien werd ten noorden van de luzernerijen en hierbij aansluitend in regels loodrecht hierop luzerne gezaaid op een oppervlakte van 10 × 21 m. Op drie tijdstippen met tussenperioden van ongeveer 2½ maand, nl. op 7 juni, 26 augustus en 3 november, werden de planten met geelnervigheid in kaart gebracht (fig. 2). Aldus werd een goed beeld verkregen van de verspreiding van het topvergelingsvirus, uitgaande van de besmette rijen.

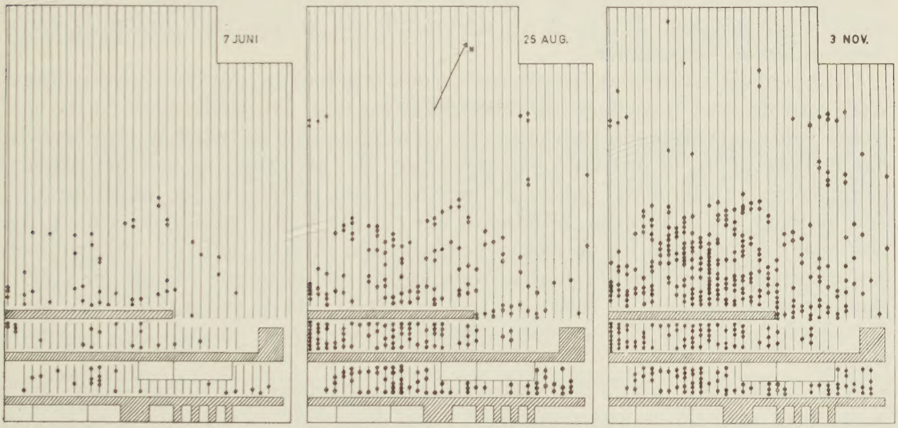

FIG. 2. Natuurlijke verspreiding van geelnervigheid in een luzerneveldje (28½ × 10 m), uitgaande van kunstmatig geïnfecteerde besmettingsstroken (gearceerd). Besmettingsstroken gezaaid en geïnfecteerd in 1957, overige luzerne gezaaid voorjaar 1958. Waarnemingen verricht op 7 juni, 28 augustus en 3 november 1958. (N.B. het veldje is in verhouding 2 × te breed afgebeeld).

Natural spread of vein yellowing in a field of lucerne (28½ × 10 m) from artificially infected rows (shaded). These rows sown and infected in 1957, remaining part of the field sown in spring 1958. Observations 7 June, 28 August, and 3 November, 1958. (N.B. the scale for the length is half that for the width).

Een tweede perceeltje met luzerne van vrijwel gelijke grootte was op ongeveer 100 m ten zuiden van het zojuist beschrevene gelegen, maar had geen besmettingsstroken met topvergelingsvirus. In dit tweede perceeltje werd op 3 november slechts bij één plant geelnervigheid waargenomen.

Aangaande het voorkomen van het geelnervigheidsymptoom in het eerste

proefveld kan worden opgemerkt, dat dit gedurende de zomermaanden steeds duidelijk zichtbaar was. In september 1958, toen veel dagen met zonneschijn en een hoge temperatuur voorkwamen, vormden de zieke planten echter weer normaal groene bladeren. Bij de laatste kartering, eind november, waren de besmette planten weer duidelijk aan de gele nerftekening op de bladeren te herkennen.

BESPREKING

Door QUANTZ & VÖLK (1954) en DE FLUITER & HUBBELING (1955) is de mening geuit, dat luzerne een waardplant van het topvergelingsvirus van de erwt is. De Duitse auteurs grondden dit op het voorkomen in luzernepercelen van planten, „die durch gestauchten Wuchs und chlorotische Aufhellung der etwas versteiften, starren Blättchen auffielen". Het gelukte hen niet, met sap van dergelijke planten een virus over te brengen. Wel vonden zij in de betrokken luzerneplanten een necrose van het floëem, een symptoom, dat zij ook in vergelingszieke erwten hadden waargenomen.

DE FLUITER & HUBBELING concludeerden tot het voorkomen van het topvergelingsvirus in luzerne uit het feit, dat met erwtebladluizen in voorjaar en voorzomer in luzernevelden in het zuidwesten des lands gevangen, gemakkelijk infectie van erwten met topvergelingsvirus tot stand kon worden gebracht. Deze auteurs vermeldden, dat zij in de desbetreffende velden geen duidelijk zieke luzerneplanten waarnamen.

Uit het in dit artikel beschreven onderzoek is gebleken, dat het topvergelingsvirus van de erwt inderdaad luzerne kan aantasten. Dit onderzoek en de verrichte veldwaarnemingen bevestigen de mening van DE FLUITER & HUBBELING, dat luzernevelden in ons land van betekenis zijn als reservoir van het topvergelingsvirus van de erwt. Onder de in Nederland voorkomende omstandigheden verwekt het in luzerne, althans in het ras Du Puits, geen symptomen, die ook maar enigermate op de ziekteverschijnselen in erwt gelijken. Integendeel, een belangrijk deel van het groeiseizoen kunnen de besmette planten er volkomen normaal uitzien. En zelfs in voor- en najaar, als de symptomen van geelnervigheid te voorschijn komen, hebben de besmette planten een normale habitus.

Naar aanleiding van de besproken proeven met de erwtebladluis moet, zoals reeds werd vermeld, worden opgemerkt, dat deze vaak met moeite in de kas op luzerne tot zuigen kan worden gebracht. Het nemen van proeven wordt hierdoor zeer bemoeilijkt. Volgens EVANS & GYRISCO (1956) zou men deze moeilijkheden kunnen verklaren doordat van deze bladluissoort de winterwaardplanten verschillen van de zomerwaardplanten. De migranten verhuizen in het voorjaar van de winterwaardplanten, zoals luzerne, naar de éénjarige zomerwaardplanten, zoals erwten. De zomergeneraties van het insekt zijn volgens deze auteurs ook in de kas doorgaans niet op luzerne te kweken, doordat luzerne dan minder aantrekkelijk voor ze is.

In werkelijkheid is het probleem echter gecompliceerder daar van *Acyrthosiphon pisum* verschillende vormen bestaan, die tot dusver morfologisch niet te onderscheiden zijn, maar b.v. wel verschillen in smaakpreferenties (HILLE RIS LAMBERS, 1947). De gecompliceerdheid blijkt ook uit het feit, dat de geelnervigheid in ons proefveld gedurende de zomer snel werd verspreid (fig. 2) en dat in dit proefveld toen grote aantallen erwtebladluizen konden worden waar-

genomen. Het is evenwel niet uitgesloten, dat ook andere bladluissoorten in de verspreiding een rol hebben gespeeld.

SAMENVATTING

Door middel van de erwtebladluis (*Acyrthosiphon pisum* HARRIS) werd het topvergelingsvirus van de erwt op luzerne (*Medicago sativa* L.) overgebracht. De besmette planten vertoonden na verloop van tijd het verschijnsel van geelnervigheid, te weten een regelmatige, lichtgroene tot gele nerftekening op de bladeren. Dit verschijnsel blijkt veelvuldig in luzernepercelen in het zuidwesten van het land (het gebied waar topvergeling in erwt algemeen voorkomt) op te treden. Het gelukte uit vier van vijf van verschillende plaatsen van West-Brabant en Zuid-Beveland afkomstige monsters geelnervige luzerne het topvergelingsvirus te isoleren.

Uit waarnemingen bij planten, groeiende in het veld en in de kas, is gebleken, dat de omstandigheden een grote invloed hebben op het te voorschijn komen van het ziektebeeld. In het voor- en najaar treedt de geelnervigheid doorgaans zeer duidelijk op; in de zomermaanden verdwijnt dit verschijnsel veelal tijdelijk bij de besmette planten en onderscheiden deze zich niet van virusvrije luzerne.

In een veldproef bleek het topvergelingsvirus, gezien het voorkomen van geelnervigheid in de loop van het seizoen, vrij snel in luzerne verspreid te worden.

SUMMARY

Tip yellows (leaf roll) virus of pea was transmitted to lucerne (*Medicago sativa* L.) by means of the pea aphid (*Acyrthosiphon pisum* HARRIS). After some months the infected lucerne plants showed a regular lightgreen to yellow discoloration of the veins and the adjacent tissue (fig. 1). No differences in growth and in shape of the leaves between infected and healthy plants were found to exist.

This disease – for which we have chosen the name of „vein yellowing of lucerne" – is very prevalent in lucerne fields in the southwestern part of The Netherlands where tip yellows is often found in peas. We succeeded in isolating tip yellows virus from four out of five samples of lucerne showing vein yellowing collected in western Brabant and Zuid-Beveland.

In carrying out transmission tests, it was found that the pea aphid often hardly sucks on lucerne in the greenhouse. Although it generally is present in large numbers in lucerne fields, it refuses to feed on lucerne after having been reared on peas for a prolonged period. This behaviour of the vector impedes the experiments. It may be due to the fact that the different generations of the insect differ in host plant requirements (EVANS & GYRISCO, 1956) but also to the fact that morphologically indistinguishable forms of the insect exist, which may differ in host plant preferences (HILLE RIS LAMBERS, 1947).

Furthermore, in our experiments seldom a 100 % infection of pea plants was obtained, even when using pea aphids which fed on a tip yellows diseased pea plant for a period of 48 hours or longer, and using five to ten aphids per test plant.

Observations on infected plants growing in the field and in the greenhouse indicate that the environment has great influence on the appearance of the vein yellowing symptom. Generally, vein yellowing occurs very strikingly during the spring-time and the late autumn but disappears in the summer months.

According to observations on the rate of occurrence of vein yellowing at different times of the year in a field experiment at Wageningen (fig. 2), it appears that tip yellows virus is capable of spreading fast in a lucerne field.

The experiments and observations described in the present paper confirm the idea expressed by DE FLUITER & HUBBELING (1955) that lucerne fields are important as a reservoir of the tip yellows virus of pea in The Netherlands.

DANKBETUIGING

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DRUK: H. VEENMAN EN ZONEN N.V. – WAGENINGEN

FIG. 1. Bladeren van luzerne (ras Du Puits) met geelnervigheid veroorzaakt door het topvergelingsvirus van de erwt. Links boven, gezond blad.

Leaves of lucerne (variety Du Puits) showing vein yellowing caused by the tip yellows virus of pea. Upper left, healthy leaf.

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INVESTIGATIONS ON WHITE CLOVER MOSAIC VIRUS[1]

Met een samenvatting:

Onderzoekingen over witte-klavermozaïekvirus

BY

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INTRODUCTION

In studying virus diseases of leguminous plants (VAN DER WANT & BOS, 1958; BOS & VAN DER WANT, 1958) we several times isolated a virus from white clover and other plants such as peas, which differed in many respects from other well-known legume viruses. This virus, designated here as white clover mosaic virus, has already been studied by VAN DER WANT (1954). However, many of its properties and much of its behaviour remained unknown.

Recently, one of the authors made a study of a virus isolate which was assumed to be common pea mosaic virus. It turned out to be a complex of viruses, white clover mosaic virus being one of them.

Since white clover mosaic virus was isolated several times from red and white clover plants from different localities in The Netherlands, and once from pea, a more detailed study of the virus was made. Although exact data on its incidence are still not available, the present paper will give an account of the results of this study.

Special attention was paid to a comparison of the Dutch virus with the American white clover mosaic virus (*Trifolium virus* 1 ZAUMEYER & WADE), which has been reported to be of complex nature (JOHNSON, 1942).

LITERATURE REVIEW

In 1947 VAN DER WANT (1954) isolated a virus from mosaic diseased white clover. Then in 1953, during his stay at Wageningen, OSWALD examined white clover plants for the presence of alfalfa mosaic virus. He isolated a virus differing from the former in not being infective to tobacco and in causing a faint systemic mosaic on beans after some initial necrosis on the inoculated leaves. This virus was presumed to be a strain of the bean yellow mosaic virus. In his studies on the bean viruses 1 and 2 VAN DER WANT (1954), however, was able to demonstrate clearly that the isolates obtained in 1947 and in 1953 differed from the bean yellow mosaic virus symptomatologically, serologically and on the basis of their morphology as studied in the electron microscope. He was the first to publish a picture of the virus particles.

Since 1935 a mosaic disease of white clover has been known in the American literature (ZAUMEYER & WADE, 1935, 1936; PIERCE, 1935). WEISS (1939)

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designated the virus as *Trifolium virus* 1 ZAUMEYER & WADE. This virus has many features in common with the Dutch isolates (VAN DER WANT, 1954). JOHNSON (1942) claimed this American virus to be of complex nature, consisting of two components, viz. "pea wilt" virus and "pea mottle" virus. These two differed slightly in symptoms, and very little in physical properties. The wilt virus could be isolated by means of inoculating the complex to cowpea (*Vigna sinensis* ENDL.) only being susceptible to the pea wilt virus. The mottle virus could be separated by means of transmission by dodder (*Cuscuta campestris* YUNCKER). This component could infect also *Antirrhinum majus* L., *Cucumis sativus* L., *Spinacia oleracea* L. and *Stellaria media* (L.) CYRILL., whereas the wilt virus was restricted to leguminous plants.

From his preliminary trials VAN DER WANT (1954) got the opinion that the Dutch isolates were not of complex nature, but nevertheless belonged to the same virus group formerly designated *Trifolium virus* 1.

Recently in Germany also QUANTZ (1956) isolated a virus from naturally infected white clover, *Medicago lupulina* and *Trifolium hybridum*, which induced symptoms in *Phaseolus vulgaris* and possessed physical properties typical for the white clover mosaic virus. QUANTZ supposed this virus to be related to the above mentioned pea wilt virus. BRANDES & QUANTZ (1957) studied the virus in the electron microscope. Morphologically their isolate was similar to the isolate of VAN DER WANT (1954). The estimated standard length of the virus was 476 mμ according to BRANDES & QUANTZ (1957).

MATERIALS AND METHODS

Four isolates of the white clover virus from different sources were studied viz.

1. WKV, isolated from white clover (isolate of VAN DER WANT, 1954),
2. ER, isolated from peas, showing necrosis; collected in 1956,
3. TRV, isolated in 1956 from red clover showing mosaic,
4. DEL, isolated in 1957 from peas with a complex of the virus together with the common pea mosaic virus. The white clover virus manifested itself by inducing a heavy wilting of infected peas in greenhouse studies during autumn when the light was very poor.

The experiments were carried out in greenhouses especially designed for virus research; those with isolate DEL, partly in a normal insect-proof greenhouse which was often fumigated. Temperatures varied from 18–23° C. The plants were grown in sterilized compost soil contained in 4 or 6 inch clay pots.

Test plants used to study the host range were infected by means of sap inoculation using carborundum as an abrasive. Susceptibility was proved by means of symptom expression, back inoculation to susceptible beans and peas, and by examination with the electron microscope.

Dodder transmission experiments were carried out with *Cuscuta campestris* YUNCKER. The parasite was grown on diseased broadbean and pea plants. After 18 days exposure to these plants dodder branches 10–20 cm long, were collected and divided into two groups, 1 – to be transferred to healthy test plants and 2 – to be tested directly for presence of the virus by means of rubbing the expressed sap onto leaves of healthy *Phaseolus vulgaris*.

In the insect transmission tests *Acyrthosiphon pisum* HARRIS, *Aphis fabae*

Scop. and *Myzus persicae* Sulz. were used. After a starvation period of one to three hours the aphids fed on peas infected with the isolate WKV for periods indicated. Then they were immediately transferred to healthy pea test plants. For each treatment, two pots containing four plants each, were tested using 20 aphids per pot. After two days, or sometimes longer, the aphids were killed by an insecticide. In another experiment the insects were observed individually during the infection feeding period and the length of this period was controlled by means of a stopwatch.

The thermal inactivation point determinations were made by placing 2 ml of freshly extracted juice of Beka beans in thin walled test tubes and immersing these in a heated water bath. Temperatures in the water bath were controlled and maintained at the desired level. After 10 minutes the tubes were rapidly cooled in tap water. Then inoculations were made. Distilled water was used in performing the dilution tests. For the ageing tests, expressed sap of Beka beans was placed in stoppered bottles on the laboratory table. At irregular intervals inoculations were made on susceptible hosts.

Various tests were repeated several times.

For preparing the electron micrographs about 50 mg of infected plant material was ground in a mortar together with 2.5 ml of distilled water. The sap thus obtained at a dilution of about 1:50, and also diluted 1:100, was atomized onto the grids covered with a thin layer of collodion by using a pyrex nebulizer. The grids were then shadow cast with palladium and viewed with a Philips electron microscope at the Physico-Technical Service for Agriculture at Wageningen by Miss C. van der Scheer and Mr. S. Henstra.

In the serology tests use was made of antiserum 1.4.15 prepared in 1951 against an isolate no longer available and antiserum 7.5.40 prepared in 1958 against the isolate DEL.

Antiserum 1.4.15 was prepared by injecting a rabbit in the ear with dialysed sap of diseased broad beans. Injections were performed four consecutive days and after an interval of three days two other days. Quantities varying from 1 to 6 ml were used. The blood was taken one week after the last injection.

Sap of French bean plants to be used for preparing the antiserum 7.5.40 was purified by centrifuging for 15 min. at 7,000 r.p.m., after which the virus-containing supernatant was concentrated in the ultracentrifuge by means of the density gradient method. The rabbit was injected in the ear for 7½ weeks at irregular intervals for a total of 12 times with quantities of virus containing liquid varying from 1½ to 3 ml. Seven days after the last injection the blood was taken.

Before performing the serological tests, the antisera were saturated by mixing with sap of healthy Beka bean (1:3), incubating the mixture at 37°C for 2 hours, cooling in a refrigerator and subsequently centrifuging at 12,000 r.p.m. for 20 min. The saturated antiserum thus obtained at a dilution 1/4 was further diluted with saline as indicated in table 3.

The sap containing virus was obtained from leaves of infected Beka beans. The leaves were frozen for two days, squeezed, filtered through cheese cloth, the sap thus obtained mixed with saline (1:3), the mixture frozen for three hours and after thawing centrifuged at 10,000 r.p.m. for 20 minutes. Dilutions as indicated in table 3 were made with saline.

The serological tests were performed according to the microreaction method

under paraffin oil developed by van Slogteren (1955). Results were read after one hour at 37° C.

Experimental results

Host range studies

We included cowpea, cucumber, spinach and some other non-leguminous plants in our host range studies to provide a comparison of our virus isolates with those of the American viruses of pea wilt and pea mottle, and also to get information whether or not our virus was of complex nature.

A scheme of the inoculations with the three Dutch isolates performed separately on several host plants and a summary of the results is given in table 1. Generally the results obtained with each of the three isolates were similar. To check the presence of virus and to determine whether differences in symptom expression might be due to the isolation of components, several back inoculations were made. In the first series of inoculations (table 1, first column) the presence of virus was checked also by means of the electron microscope (cf. also electron microscopy).

In peas, French bean, cowpea, red clover, crimson clover and in broad bean infection was evident both on the basis of symptom expression and electron microscopy. In most cases large numbers of particles characteristic of the white clover mosaic virus were present. Generally in white clover it was difficult to see symptoms. However, by means of the electron microscope infection could easily be proven. In all these plants, back inoculations supported these primary results (column 2), being the same for cowpea and the other mentioned plant species. From lucerne without symptoms, back inoculations repeated twice, could not reveal infection or presence of the virus. In a separate trial to test lucerne again as a possible host plant, the same results were obtained.

In spinach (*Spinacia oleracea* L.), *Gomphrena globosa* L. (detached leaves in petri dishes), sweet william (*Dianthus barbatus* L.) and carnation (*D. caryophyllus* L.) we could not detect infection in any way.

In cucumber, no. 10 of the first series of host plants in table 1, we could not prove infection by means of symptoms or the electron microscope. In doing back inoculations to Beka beans and Mansholt peas, virus appeared to be present, symptoms suggesting the inciting agent to be the white clover mosaic virus. Using the sap from this Beka beans we again were able to demonstrate the presence of the white clover mosaic virus by inoculating Beka beans, Mansholt peas, cowpea and broad bean. By using the same sap, in this case all three isolates were infectious to cucumber, inducing numerous small yellow spots on the inoculated cotyledons and definite yellow, diffuse, systemic flecks on the higher leaves (fig. 7). The virus also could be recovered from these cucumber plants in the same way as could be done from the other plants of this series (table 1, last column).

In this way the results obtained with cucumber (no. 10) were the same as those with cowpea (no. 4). Via cowpea, cucumber also could be infected. The only difference observed in the last series of back inoculations (last column) was a delayed appearance of symptoms in the Beka beans and in the Mansholt peas when using the infectious sap from cucumber (no. 10) in comparison with the virus from cowpea (no. 4). Similar results were obtained in a test with isolate WKV from infected cowpea and cucumber inoculated to Mansholt and

Servo peas. This was good evidence that the white clover mosaic virus can pass through cowpea and infect cucumber and vice versa.

The results of host range experiments obtained separately in a slightly different way with the isolate DEL, agreed quite well with those mentioned above. The only difference might be the infection of lucerne with isolate DEL which produced an inconspicuous mottle, being not conclusive, however. Transferring this isolate from cowpea and cucumber to beans and peas as test plants, these gave similar results to those obtained with the other three isolates.

The results of these experiments indicate that the white clover mosaic virus can go to cowpea as well as to cucumber. This is in contrast with the results published by JOHNSON (1942). The fact, that both these plant species can be infected with our isolates, suggests a similarity of our virus with the white clover mosaic virus of JOHNSON as a whole (wilt and mottle components together). That all four isolates of the virus, studied by us, did not go to spinach might be due to a difference in variety of this test plant.

Bean varietal reaction. Eight bean varieties were tested for their reaction to the WKV isolate of the white clover mosaic virus. All varieties were susceptible. These included the dwarfbeans Citroengele and Noordhollandse Bruine, the dwarf French (snap) beans Hinrich's Reuzen, Roem van Holland, and Processor, the pole snap bean Rentegevers zonder draden, the pole bean for slicing Verschoor and the haricot bean Kievitsboon. For the symptoms produced cf. page 96.

Two varieties of runner beans (*Phaseolus coccineus* L.), Emergo and Prijswinner, were tested; both turned out to be immune.

Pea varietal reaction. Twelve pea varieties were tested for their reaction to the WKV isolate of the virus. All varieties were susceptible. These included the varieties Alaska, Big Ben, Erickson Perfection, Eroica, Juweel, Kelvedon Wonder, Koroza, Rondo, Servo, Vares, Venlose Lage and Wyola. Another series of 15 pea varieties tested against the DEL-isolate demonstrated the susceptibility of all these varieties.

Symptomatology

By mechanical inoculation to 14 species of test plants (table 1) the three isolates WKV, ER and TRV were compared on the basis of the symptoms they produced in susceptible plants. They did not differ in host range (p. 92). According to the symptoms produced, they hardly showed differences. On Beka beans TRV had a tendency towards a somewhat longer incubation period. WKV appeared to be the most virulent of the isolates. Isolate DEL was studied in a separate test. The symptoms in various hosts due to the white clover mosaic virus in general will be described here.

1. Pea (*Pisum sativum* L.): About six days after inoculation the inoculated leaves wilt and the younger foliage is epinastic. Generally they also show some vein clearing and afterwards a diffuse mottling consisting of slightly darker or sometimes light, small, yellow spots. About eight days after inoculation the stem shows a grayish discoloration and the sides of the stem are sunken. Affected plants are often stunted, but they may recover to some extent. The wilt often progresses upwards (fig. 1). Then the plants die, sometimes giving rise to new outgrowth of basal branches. These develop the diffuse mottle mentioned

TABLE 1. Scheme of inoculations, back-inoculations and results obtained with the Dutch isolates WKV, ER, and TRV of the white clover mosaic virus.

Schema van inoculaties, teruginoculaties en resultaten verkregen met de Nederlandse isolaties WKV, ER en TRV van het witte-klaver-mozaïekvirus.

Date of inoculation *Inoculatiedatum*	25/3		8/4		16/5		9/6	
Plants tested *Getoetste planten*	Reaction[1] *Reactie*	EM[2]	Plants tested[4] *Getoetste planten*	Reaction *Reactie*	Plants tested *Getoetste planten*	Reaction *Reactie*	Plants tested *Getoetste planten*	Reaction *Reactie*
1. pea var. Eroïca *erwt var. Eroïca*	+	+	Beka	+				
			Mansholt	+				
2. pea var. Mansholt's Pluk *erwt var. Mansholt's Pluk*	+	+	Beka	+				
			Mansholt	+				
3. bean var. Beka *boon var. Beka*	+	+	Beka	+				
			Mansholt	+				
4. cowpea var. 37 c–6 *Vigna sinensis*	+	+	Beka	+	Beka	+	Beka	+
			Mansholt	+	Mansholt	+	Mansholt	
					cowpea (Black eye)	?	cowpea 37 c–6	+
					cucumber	+	cowpea (Black eye)	?
					broad bean	+	Beka	+
							Mansholt	+
							cowpea 37 c–6	+
							cowpea (Black eye)	?
							Beka	+
							Mansholt	+
							cowpea 37 c–6	+
							cowpea (Black eye)	?
5. red clover *rode klaver*	+	+	Beka	+				
			Mansholt	+				
6. white clover *witte klaver*	(+)	+	Beka	+				
			Mansholt					
7. crimson clover *inkarnaat klaver*	+	+	Beka	+				
			Mansholt	+				
8. lucerne *luzerne*	—	—	Beka	?				
			Mansholt	?				
					Beka	—		
					Mansholt			

9. broad bean *tuinboon*	+	+	Beka	.				
			Mansholt	+				
10. cucumber *komkommer*	—	—	Beka	+	Beka	+	Beka	.
			Mansholt	+	Mansholt	+	Mansholt	.
					cowpea (Black eye)	?	cowpea 37 c–6	+
					cucumber	+	cowpea (Black eye)	?
					broad bean	?	Beka	+
							Mansholt	+
							cowpea 37 c–6	+
							cowpea (Black eye)	?
							Beka	+
							Mansholt	+
							cowpea 37 c–6	+
							cowpea (Black eye)	?
11. spinach *spinazie*	—	—	Beka	?	Beka	—		
					Mansholt	—		
					red clover	—		
			Mansholt	?	Beka	—		
					Mansholt	—		
					red clover	—		
12. Gomphrena globosa [3]	—	—	Beka	?	Beka			
					Mansholt	—		
					red clover	—		
			Mansholt	?	Beka	—		
					Mansholt	—		
					red clover	—		
13. sweet william *duizendschoon*	—	—	Beka	?	Beka	—		
					Mansholt	—		
					red clover	—		
			Mansholt	?	Beka	—		
					Mansholt	—		
					red clover	—		
14. carnation *anjer*	—	—	Beka	?	Beka	—		
					Mansholt	—		
					red clover	—		
			Mansholt	?	Beka	—		
					Mansholt	—		
					red clover	—		

[1] + symptoms typical for the white clover mosaic virus
symptomen karakteristiek voor het witte-klavermozaïekvirus

— no symptoms
geen symptomen

? symptoms questionable
symptomen onzeker

[2] presence of the virus checked by means of the electron microscope
aanwezigheid van het virus gecontroleerd met de elektronenmicroscoop

[3] detached leaves on moist blotting paper in petri dishes
afgesneden bladeren op vochtig vloeipapier in petrischalen

[4] for convenience only name of variety used
gemakshalve alleen de naam van het ras genoemd

above (fig. 2). All varieties of pea tested were susceptible. There was only a slight difference in symptom expression. The pea varietal reaction test was performed in the greenhouse. Simultaneously a parallel series was placed in a conditioned cabinet with poor light (four T.L. tubes 60 Watt per m²) and a relatively high temperature (18–20°). In this series all varieties wilted within one or two weeks.

2. Bean (*Phaseolus vulgaris* L.), variety Beka: The first reaction could be observed about six days after inoculation. It consisted of a beginning epinasty of the inoculated primary leaves with often diffuse chlorotic spots of about two to three mm in diameter (fig. 3A) showing a tendency to become necrotic along the edge or in the centre (fig. 3B). Moreover these primary leaves show a local grayish discoloration of the big veins (fig. 3B) and petioles. After some time this could be also observed in the lower part of the stem and incidentally in the petioles of higher leaves. As studied anatomically this discoloration appeared to be due to an irregularly distributed formation of dark brown gum associated with some necrosis in the parenchyma. Especially big groups of cells in the regions between the vascular bundles are filled with gum. To some extent this holds also for the parenchyma between the xylem and in the pericycle, whereas here also more necrosis is apparent. The systemic symptoms, appearing about eight days after inoculation in the trifoliate leaves, consisted of local chlorotic discolorations of the veins and neighbouring interveinal tissue. This symptom is mostly characterized by a local, irregularly bordered clearing of the tiny reticulated veinlets (fig. 4A). It gave these leaves a blotted mosaic-like appearance, varying from regularly scattered small asteroid spots to large, irregularly shaped, local areas with a yellow-green, translucent colour. In the latter case the leaf often was somewhat malformed. The smaller spots could also coalesce. In this way the abnormality extended to the entire surface of the leaf (fig. 4B). With age the plants more or less recovered, showing a diffuse mosaic in the higher leaves. The latter symptom could easily be confused with those of bean yellow mosaic.

In Beka bean TRV is distinguished from the other three isolates by a later appearance of the systemic symptoms. After inoculation with this isolate the first trifoliate leaf was generally almost normal, with only a few small, asteroid spots. The younger leaves, however, produce the normal type of symptoms. One month after inoculation there was no difference in symptom expression between TRV and the other isolates.

In the dilution tests it appeared like the slow type of systemic infection was due to a low concentration of the virus in the inoculum.

Thus symptoms could vary from a severe type of reaction to a mild type. The former consisted of serious necrosis in the petiole of the inoculated primary leaves and a systemic vein clearing beginning in the first ternate leaf. The latter type was characterized by absence of necrosis, presence of some areas of systemic vein clearing in the first ternate leaf and a more general vein clearing in subsequent leaves. Finally even an initial visible reaction in the second ternate leaf was possible.

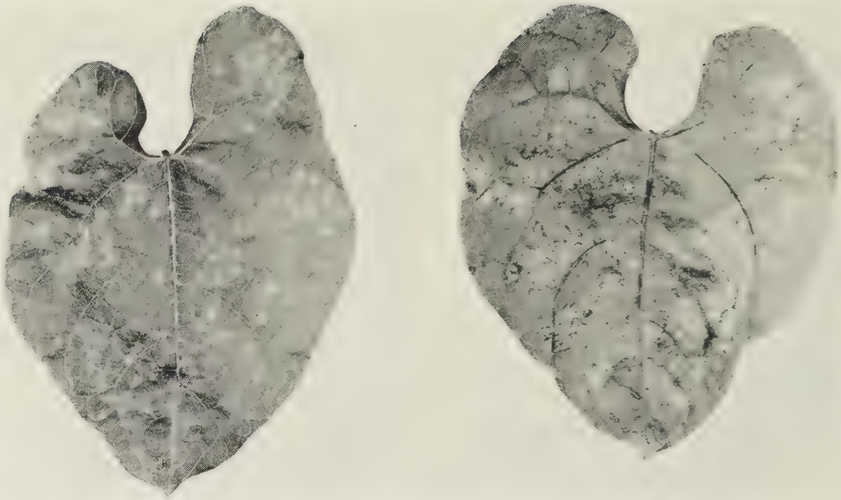
In the bean varietal reaction test eight bean varieties reacted similarly with only slight differences in severity of symptoms. All varieties showed the characteristic grayish internal discoloration of the petioles of the inoculated primary leaves and systemic vein clearing. Seven weeks after inoculation the plants were

FIG. 1. Wilting in peas (*Pisum sativum* L.), var. Eroica, inoculated with the white clover mosaic virus. Right, healthy plants.

Verwelkingsverschijnselen in erwt (Pisum sativum *L.*), *ras Eroica, na inoculatie met het witte-klavermozaïekvirus. Rechts, gezonde planten.*

FIG. 2. Diffuse mottle in peas (*Pisum sativum* L.), var. Fertilas, inoculated with the white clover mosaic virus.

Diffuse gevlektheid in erwt (Pisum sativum *L.*), *ras Fertilas, na inoculatie met het witte-klavermozaïekvirus.*



A B

FIG. 3. Primary leaves of bean (*Phaseolus vulgaris* L.), var. Beka, inoculated with the white clover mosaic virus. A, 5 days after inoculation; B, 14 days after inoculation (After VAN DER WANT, 1954).

Primaire bonebladeren (Phaseolus vulgaris *L.*), *Ras Beka, geïnoculeerd met het witte-klavermozaïekvirus. A, 5 dagen na inoculatie; B, 14 dagen na inoculatie. (Naar van der Want, 1954).*

A B

FIG. 4. Leaves of bean (*Phaseolus vulgaris* L.), var. Beka, showing systemic symptoms eleven days after inoculation of the primary leaves. A, Local irregular clearing of the tiny reticulated veinlets; B, more general vein clearing. (B, After VAN DER WANT, 1954.)

Bonebladeren (Phaseolus vulgaris *L.*), *ras Beka, met spreidsymptomen elf dagen na inoculatie van de primaire bladeren. A, Plaatselijke, onregelmatig begrensde, chlorotische verkleuring van de fijne nerven; B, meer algemene nerfverkleuring. (B, Naar van der Want, 1954.)*

FIG. 5. Leaves of crimson clover (*Trifolium incarnatum* L.) showing a diffuse mosaic caused by the white clover mosaic virus, artificial infection. Right, healthy leaf.

Bladeren van inkarnaatklaver (Trifolium incarnatum *L.*) *met een diffuus mozaïek na kunstmatige infectie met het witte-klavermozaïekvirus. Rechts, gezond blad.*

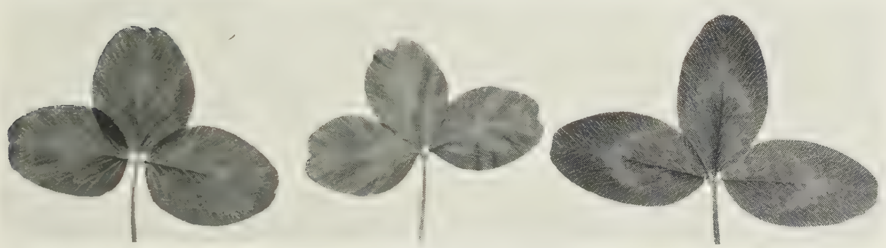


FIG. 6. Leaves of red clover (*Trifolium pratense* L.) showing mosaic symptoms caused by the white clover mosaic virus, artificial infection. Right, healthy leaf.

Bladeren van rode klaver (Trifolium pratense *L.*) *na kunstmatige infectie met het witte-klavermozaïekvirus. Rechts, gezond blad.*

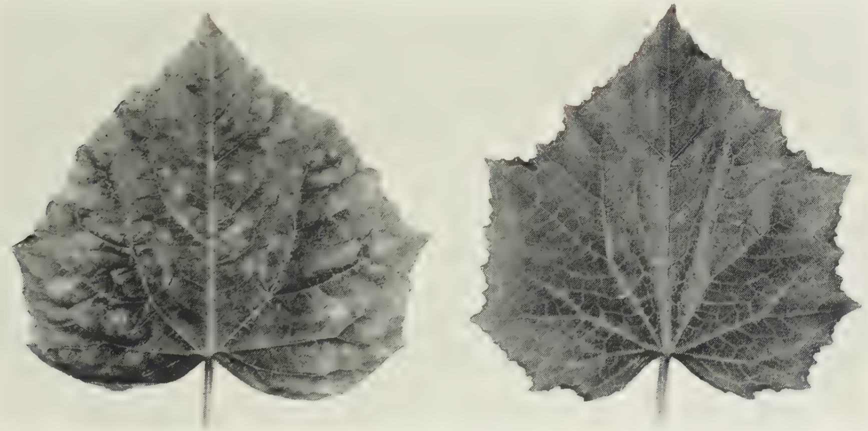


FIG. 7. Systemic, diffuse yellow spots on the secondary leaves of cucumber (*Cucumis sativus* L.) artificially infected with the white clover mosaic virus. Right, healthy leaf.

Systemische, diffuus gele vlekjes op de secondaire bladeren van komkommer (Cucumis sativus *L.*) *na kunstmatige infectie met het witte-klavermozaïekvirus. Rechts, gezond blad.*

FIG. 8. Electron micrograph of the particles characteristic for the white clover mosaic from sap of pea. Preparation made by the spray method. Magnification × 20,000. (Photograph made by the Physico-Technical Service for Agriculture, Wageningen).

Elektronenmicroscopische foto van in erwtesap voorkomende deeltjes, welke karakteristiek zijn voor het witte-klavermozaïek. Vergr. 20.000 ×. (Foto gemaakt door de Stichting Landbouw Fysisch-Technische Dienst, Wageningen).

only slightly stunted. The younger leaves were somewhat smaller than normal, and showed a diffuse coarse mottle and an irregular surface. Some younger pods developed small sunken spots.

3. In broad bean (*Vicia faba* L.), variety "Driemaal Wit": Systemic symptoms in the younger leaves were visible about eight to nine days after inoculation. The consisted of somewhat diffuse ring-like spots and sometimes, especially with isolate ER, also of some local, faint, irregularly bordered vein clearing. After some time, a mild mosaic including some slight vein clearing within the light green areas was predominant in the upper leaves.

4. Cowpea (*Vigna sinensis* Endl.) variety 37 c–6: Small chocolate-brown necrotic lesions developed on the upperside of the primary leaves six days after inoculation. The systemically infected trifoliate leaves showed an irregular mosaic pattern. The "Black eye" cowpea did not show the brown local lesions on the inoculated primary leaves, but in a repeated trial with the isolate WKV faint chorotic rings, measuring about 1.5 mm, were observed. This variety also showed a faint systemic mosaic.

5. In white clover (*Trifolium repens* L.) the symptoms were hardly visible. In the experiments a number of plants developed a faint diffuse mottle. Since clovers genetically are very variable, their reaction to viruses may also show a great variation.

6. Crimson clover (*Trifolium incarnatum* L.) generally showed an irregular, diffuse mosaic (Fig. 5).

7. Red clover (*Trifolium pratense* L.) showed the first clear symptoms about eight days after inoculation. The syndrome varied from an irregular diffuse mottle, sometimes in combination with a clearing of veins, to a distinct mosaic more or less parallel to the side-veins (fig. 6). Severely diseased leaves generally had an irregular surface and curled edges.

8. Cucumber (*Cucumis sativus* L.) showed numerous yellow-green spots on the inoculated cotyledons five days after inoculation. Later diffuse yellow spots on the secondary leaves developed (fig. 7).

Infectivity tests

Although the research on white clover mosaic virus was carried out very carefully we sometimes got infection of a single control plant. Thus, the idea arose that the virus might be very contagious. Therefore we made some tests with Beka beans to study the infectivity of the virus in more detail. The results will be briefly summarized.

1. *a.* A diseased leaf was rubbed very gently over the upper surface of the leaves of healthy plants, without extra pressure. Results: 1/8 (1 plant infected of 8 plants tested); typical symptom reaction.
   b. do, but with some pressure of the finger. Results: 7/8; mild reaction.
   c. do, but with heavy pressure, both leaves damaged visibly. Results: 8/8; severe reaction.
   d. Finger rubbed over diseased leaf and then over healthy leaves. After each plant, rubbing over diseased leaf repeated. Results: 2/8; very mild reaction.
2. *a.* Leaves inoculated by means of rubbing with the finger without the use of carborundum. Results: 8/8; very severe reaction.

b. Leaves inoculated with finger still wet after having used it for method 2a and cleaned by rinsing with tapwater. Results: 7/8; rather severe reaction.

3. Plants inoculated in a normal way (carborundum) with material of an infected leaf of French bean dried on the laboratory table for 12 days and afterwards ground in a mortar with some water. Results: 3/8; rather mild reaction.
4. Normal inoculation with sap of a diseased plant and carborundum. Results: 3/3: very severe and typical symptoms.

The results obtained indicate that the virus is rather contagious and in experiments should be handled carefully to prevent unintentional spread.

To check the possibility of transmission in the field by contact of plants the following experiment was made. Two pots containing a total of seven diseased French bean plants were surrounded by ten pots, each containing four healthy plants. At one side from a distance of about 60 cm during the first 19 days one hour per day, except on Sunday, by means of a small oscillating fan, air was blown over this group of plants causing mutual contact. One month after the beginning of the trial all 34 test plants which had survived showed symptoms of white clover mosaic virus infection.

Transmission by dodder

Successful transmission of the virus by means of dodder was demonstrated in a preliminary trial with the isolate DEL. Therefore with the isolate WKV a more extensive test was carried out.

Dodder stems, after having parasitized diseased broad beans and peas, were transferred to healthy broad beans and peas, five pots each. The dodder was tested for presence of the virus by rubbing expressed sap onto the leaves of Beka beans. In one out of eight plants a positive reaction was obtained. After two weeks with the same dodder this test was repeated resulting in a very conclusive WKV reaction in three out of eight Beka plants. The broad bean and pea plants, parasitized by the infectious dodder in the meantime began showing virus symptoms. These were not conclusive, however, due to abnormal growth of the plants. Two back inoculations onto Beka bean plants indicated the presence of the white clover mosaic virus. Back inoculations $4\frac{1}{2}$ weeks after transferring the dodder to the plants concerned, were more conclusive (from peas seven out of seven Beka plants with very distinct symptoms) than three weeks after inoculation with dodder (from peas one out of seven Beka plants with a distinctive reaction, from broad bean four out of seven Beka plants with a questionable reaction).

The results of these studies indicate that dodder can become infected with white clover mosaic virus by parasitizing diseased plants, and that it can transmit the virus to healthy plants.

Transmission by insects

No transmission was obtained in two experiments with two types of the green pea aphid, with the green peach aphid and the black bean aphid after infection feeding periods of 5, 10, 15 and 30 minutes on peas used as test plants. To check these results from several test plants back inoculations were made, without results however.

To make results more meaningful in a third experiment with the green pea aphid and the green peach aphid the feeding periods were controlled by means of a stopwatch. With the green pea aphid 5 insects fed 15 sec., 105 sec., 150 sec. and 195 sec., respectively. With the green peach aphid 1 insect fed 7 sec. 4–10 sec., 4–15 sec., 2–20 sec., 1–25 sec., 1–35 sec., 1–40 sec., 4–60 sec., 1–70 sec., respectively. No symptoms were induced in the peas used as test plants. Likewise no transmission was obtained in a similar experiment with the green peach aphid when the observed infection feeding periods were varied from one to five minutes on healthy Beka beans used as test plants.

These results indicate that no insect transmission takes place with the aphid species tested. This is not in agreement with the results formerly obtained by one of the authors (VAN DER WANT, 1954): even at that time results were very poor, however, transmission being obtained with only 2 out of 32 *Myzus persicae* and none out of 16 *Aphis fabae*.

Physical properties

The results of the thermal inactivation tests and of the dilution tests were quite consistent, as are the data published in the literature on the white clover mosaic virus (table 2). Several tests with the same isolate produced different results, e.g. isolate WKV once was inactivated between 58° and 60° C, once between 60° and 62° C and in another test between 65° and 70° C. In this way certain differences between the isolates obtained in one test often differed from those obtained in another test. The highest inactivation point observed was between 65° and 70° C. In this case the reaction of the Beka beans used as test plants was very weak, the incubation period being about twice as long as normal, whereas the primary reaction failed entirely (see p. 96). Presumably this slow type of reaction is due to a very low concentration of the virus in the inoculum.

In the same way the results of dilution tests varied from complete inactivation at a dilution of 10^{-4} to an inactivation at a dilution higher than 10^{-9}. The latter dilution endpoint is exceptionally high. In the literature data on the dilution endpoint of this virus range from about $1 : 2 \times 10^3$ to 10^{-6} (table 2).

Apparently in these experiments the virus concentration in the original

TABLE 2. Physical properties of the white clover mosaic virus according to literature.
Fysische eigenschappen van het witte-klavermozaïekvirus naar gegevens uit de literatuur.

References *Literatuur*	Thermal inactivationpoint *Thermale inactiveringspunt*	Dilution endpoint *Verdunningseindpunt*	Days of ageing in vitro *Houdbaarheid in vitro in dagen*
ZAUMEYER & WADE (1955) . . .	58°–65°C	± 1/2000	28–32 hrs
PIERCE (1935)	< 58°C		5–7
JOHNSON (1942) "pea mottle" . .	60°–62°C	1/10,000–1/100,000	>31
"pea wilt" . . .	58°–60°C	1/100,000–1/1,000,000	>31
VAN DER WANT (1956) isolate 1947	60°–65°C	1/100,000–1/1,000,000	
isolate 1953	60°–65°C	> 1/10,000	> 3
QUANTZ (1956)	> 58°C	1/100,000–1/1,000,000	>20

material played an important role in determining the thermal inactivation and dilution endpoints.

The three isolates WKV, TRV and DEL tested for ageing *in vitro* were still infective after 99 days. Then the stored sap of Beka bean possessed an exceptionally offensive odor. All three isolates were still active in dry leaves from Beka bean, 13 days after harvesting. Isolate WKV was still active after 21 days, but not after 29 days.

Electron microscopy

Morphologically the particles observed from diseased plants (fig. 8) were quite the same as those pictured by VAN DER WANT (1954) and BRANDES & QUANTZ (1957). The peak of the frequency curves generally falls in the column of 475 mμ. This is in agreement with the standard length of 476 mμ estimated by BRANDES & QUANTZ. As already pointed out in a recent paper (HAGEDORN, BOS & VAN DER WANT, 1959) our length measurements are not yet accurate enough to furnish a reliable basis for exact comparison with the results obtained by the above German authors.

In host range studies with the isolate WKV, ER and TRV, we tested the plants as a source of inoculum and afterwards also the inoculated plants for the presence of the virus. The results are summarized in column 1 of table 1. Particles could be seen from all plant species, except cucumber, which appeared to be infected as shown by symptom expression and back inoculation. However, pea plants back inoculated with sap from cucumber showing symptoms, also produced particles visible with the electron microscope. In most cases they could be easily detected even in white clover with very faint or no symptoms. Generally large quantities of particles were present in susceptible plants (fig. 8). No particles were found in the non-leguminous plants studied.

In morphology and particle lengths distribution no differences could be observed between the three isolates mentioned above and isolate DEL, or between the host plants. Cowpea gave similar results to those obtained with the other host plants. Likewise pea plants inoculated with sap of infected cucumber contained particles with the same morphology and distribution curve as the other hosts. This furnished further evidence of the ability of the white clover mosaic virus as a whole to infect cowpea as well as cucumber. The existence of special components as published by JOHNSON (1942) could not be shown.

In preparing the electron micrographs we used the spraymethod (p 000). This resulted in a clear tendency of the particles to break into smaller parts. Often some of these smaller parts, having the total length of the assumed particle unit, were lying closely together. In applying this method to the red clover vein-mosaic virus a similar effect was found (HAGEDORN, BOS & VAN DER WANT, 1959).

Serology

VAN DER WANT (1954) prepared three antisera against the isolates 1947 and 1953 of the virus. He was able to demonstrate a specific reaction of these sera with the white clover mosaic virus. No serological relation could be observed between the white clover mosaic virus on one hand, the alfalfa mosaic virus and the bean yellow mosaic virus on the other. One of these antisera (1.4.15) was

used to test our isolates and also to compare it with a freshly prepared antiserum (7.5.40) against the isolate DEL. The results of these tests are summarized in table 3. The precipitate was clear and fluffy. In this test the four isolates appeared to react similarly.

The white clover mosaic virus appears to have some characters in common with potato virus X. Therefore the antisera were tested reciprocally but no serological relation could be shown.

HAGEDORN, BOS & VAN DER WANT (1959) could not show a serological relationship between the white clover mosaic virus and the red clover vein-mosaic virus.

DISCUSSION

The results obtained with the four Dutch isolates of the white clover mosaic virus indicate that they do not differ fundamentally.

As to symptoms, experimental host range and physical properties no substantial differences appeared to exist between the Dutch virus and the white clover mosaic viruses described in the United States of America and in Germany. So the most authentic name should be the "white clover mosaic virus" or *Trifolium virus* 1 ZAUMEYER & WADE. The symptoms in French bean are especially characteristic and of diagnostic value.

Concerning the complex nature of this virus, as was assumed by JOHNSON (1942), the results of the present authors agree with those of JOHNSON in so far that both cowpea and cucumber are susceptible to the virus. However, we were unable to show the existence of two components of the virus in The Netherlands. This was clearly demonstrated by means of tests repeated several times as back inoculations and electron microscopy. Our results indicate that our virus is in many ways the same as that described by JOHNSON, and that the Dutch virus is not of complex nature. Our results suggest that this holds true also for the American virus. Also BRANDES & QUANTZ (1957), who studied a German isolate of the virus in the electron microscope, could not reveal a complex nature of this German white clover mosaic virus.

The high infectivity of the virus, transmission even by contact, together with the high resistance to dilution, to ageing *in vitro*, and also to some extent to drying in detached infected leaves, and the presence of particles in infected susceptible plants in high concentrations, make the virus a potentially dangerous one. In experiments the white clover mosaic virus has to be handled carefully. These facts, and the observations made in our inventory studies of the viruses of leguminous crops, make it highly probable that we are dealing with a widespread virus. The fact that the virus is not or rarely found in annual crops such as peas and beans might be due to several reasons. In peas the symptoms are not very characteristic. In French beans some weeks after artificial inoculation the diffuse mosaic symptoms often suggest the presence of bean yellow mosaic virus. So it might be that possible infections of these crops with this virus are not recognized. Presumably the virus is not important in these crops because of absence of insect vectors. This virus may be of importance, however, in meadows and lawns. Possibly the virus is spread by mowing and may be by the grazing of cattle.

The results of insect-transmission tests obtained thus far suggest the absence of important insect vectors. Positive transmissions with two out of 32 green

TABLE 3. Reaction of two antisera with four Dutch isolates of the white clover mosaic virus.
Reactie van twee antisera met vier Nederlandse isolaties van het witte-klavermozaïekvirus.

Dilutions of sap of Beka beans *Verdunningen sap van Beka bonen*		Antiserum 1.4.15 *Antiserum 1.4.15*				Antiserum 7.5.40 *Antiserum 7.5.40*				Normal serum [1] *Normaal serum* [1]	Saline *Fysiologisch zout*
		1/4	1/16	1/64	1/256	1/4	1/16	1/64	1/256		
isolate WKV *isolatie WKV*	1/4	+++ [2]	++	+	—	+++	++	+	—	—	—
	1/16	+	+	—	—	++	+	—	—	—	—
	1/64	— [3]	—	—	—	—	—	—	—	—	—
isolate ER *isolatie ER*	1/4	++	+	—	—	+++	++	++	+	—	—
	1/16	+	—	—	—	++	+	+	—	—	—
	1/64	—	—	—	—	—	—	—	—	—	—
isolate TRV *isolatie TRV*	1/4	+++	++	+	—	+++	++	+	—	—	—
	1/16	+	+	—	—	—	—	—	—	—	—
	1/64	—	—	—	—	—	—	—	—	—	—
isolate DEL *isolatie DEL*	1/4	+++	++	+	—	+++	++	+	—	—	—
	1/16	+	—	—	—	++	+	—	—	—	—
	1/64	—	—	—	—	—	—	—	—	—	—
healthy *gezond*	1/4	—	—	—	—	—	—	—	—	—	—
	1/16	—	—	—	—	—	—	—	—	—	—
	1/64	—	—	—	—	—	—	—	—	—	—

[1] All dilutions tested (1/4 to 1/1024) reacted similarly.
Alle getoetste verdunningen (1/4 tot 1/1024) reageerden gelijk.

[2] The number of + signs is an indication for the quantity of precipitate.
Het aantal + tekens is een maat voor de hoeveelheid precipitaat.

[3] No precipitate.
Geen precipitaat.

pea aphids (and none out of 16 black bean aphids) formerly obtained by one of the authors (VAN DER WANT, 1954) being not entirely conclusive in view of the ease of contamination.

In the electron microscope morphologically the particles, characteristic for white clover mosaic, have the same appearance as those of potato virus X disease, as was already pointed out by BRANDES & QUANTZ (1957). According to these authors the only difference is the accurate length, being 476 mμ for the white clover mosaic virus and 515 mμ for potato virus X. Both viruses appear to have many features in common, e.g. high dilution endpoint and thermal inactivation point, high resistance to ageing *in vitro*, resistance to drying in the leaf, and probably the absence of aphid vectors. Potato virus X could also be transmitted mechanically to the leguminous host *Trifolium incarnatum*. *Nicotiana tabacum* and *Gomphrena globosa*, being useful indicators for potato virus X, however, were not infected by white clover mosaic virus after mechanical inoculation. In crimson clover no cross protection could be demonstrated. Both viruses also appeared to differ serologically.

It is interesting to emphasize the fact that the white clover mosaic virus appears to have an experimental host range not restricted to leguminous plants. Probably the same applies for the natural host range. More and more evidence is obtained that this holds for other so called legume viruses as well.

SUMMARY

Four isolates of a white clover mosaic virus found in The Netherlands were studied. The virus was compared with the white clover mosaic virus (*Trifolium virus* 1 ZAUMEYER & WADE) described in America.

No evident differences between the four Dutch isolates could be shown.

In the host range studies the legumes *Phaseolus vulgaris* L. (all eight varieties tested), *Pisum sativum* L. (all twelve varieties tested), *Trifolium incarnatum* L., *T. pratensis* L., *T. repens* L., *Vicia faba* L. and *Vigna sinensis* ENDL., and the non-leguminous species *Cucumis sativus* L. were susceptible. No infection was obtained in the legume *Phaseolus coccineus* L. (both two varieties tested) and the non-leguminous species *Dianthus barbatus* L., *D. caryophyllus* L., *Gomphrena globosa* L. and *Spinacia oleracea* L., and presumably not in the legume *Medicago sativa* L.

The symptoms in susceptible host plants are described in detail.

The virus is very infectious. Transmission even occurs by contact.

Transmission by means of *Cuscuta campestris* YUNCKER was confirmed. The presence of virus in this parasite could be demonstrated.

In insect transmission tests with *Acyrthosiphon pisum* HARRIS, *Myzus persicae* SULZ. and *Aphis fabae* SCOP., even after a long starvation period and short infection feeding periods, no transmission could be obtained.

Results of physical property tests were quite inconsistent, but generally in agreement with data already published. The thermal inactivation point varied between 58°–60° C to 65°–70° C. The dilution endpoint varied from above 10^{-4} to lower than 10^{-9}, which in the latter case is extremely low. In the ageing *in vitro* tests the virus was still active after 99 days storage. In air-dried leaves the virus was active once after 21 days, but not after 29 days.

As studied in the electron microscope the occurrence of threadlike particles

measuring about 475 $m\mu$, generally occurring in large quantities, was confirmed.

A new antiserum was tested together with one prepared earlier by VAN DER WANT (1954). All four isolates of the virus reacted similarly.

The virus studied appeared to be very similar to the American "white clover mosaic virus" (ZAUMEYER & WADE, 1935; PIERCE, 1935) and the German "Weisskleevirus" (QUANTZ, 1956). On the basis of host range studies, symptomatology and electron microscopy the assumption of JOHNSON (1942) as to the complex nature of this virus could not be confirmed.

The white clover mosaic virus turned out to have many features in common with the potato virus X. In certain respects, however, these viruses differ fundamentally.

SAMENVATTING

Het in Nederland reeds door VAN DER WANT (1954) aangetoonde wittoklavermozaïekvirus werd bestudeerd aan de hand van vier isolaties (WKV uit witte klaver, ER uit erwt, TRV uit rode klaver en DEL uit een complex met erwtemozaïekvirus in erwt). Het Nederlandse virus werd vergeleken met het Amerikaanse „white clover mosaic virus" (*Trifolium virus* 1 ZAUMEYER & WADE). Dit virus zou volgens JOHNSON (1942) bestaan uit een complex van het „pea wilt" virus (te isoleren met *Vigna sinensis* ENDL.) en het „pea mottle" virus (te isoleren met niet-leguminosen zoals komkommer, of door overbrenging met *Cuscuta campestris* YUNCKER).

De vier bestudeerde Nederlandse isolaties vertoonden geen duidelijke verschillen.

In het waardplantonderzoek werd de vatbaarheid van een aantal plantesoorten nagegaan op grond van de symptomen, teruginoculatie en met behulp van de elektronenmicroscoop. De resultaten zijn weergegeven in tabel 1. Door middel van teruginoculatie kon worden aangetoond dat *Vigna* en komkommer beide met hetzelfde virus waren besmet als de andere vatbare plantesoorten. Zo was het ook mogelijk uitgaande van *Vigna* komkommer te infecteren en omgekeerd.

Acht bonerassen en twaalf erwterassen werden getoetst. Ze bleken alle vatbaar in tegenstelling met de twee getoetste pronkbonerassen.

De ziekteverschijnselen, die de vatbare plantesoorten in de kas vertoonden, werden uitvoerig beschreven. In erwten treedt vooral een aan de basis van de plant beginnende verwelking op de voorgrond (fig. 1). Vooral bij weinig licht en een relatief hoge temperatuur sterven de planten vaak geheel af. Is dit niet het geval dan vertonen de hogere bladeren meestal een diffuse gevlektheid (fig. 2). In bonen (*Phaseolus vulgaris* L.) is de reactie zeer karakteristiek en van diagnostische betekenis. De eerste symptomen bestaan vaak uit kleine chlorotische vlekjes op de geïnoculeerde, enigszins epinastische primaire bladeren (fig. 3A). Na enige tijd vertonen deze vlekjes de neiging langs de rand en/of in het centrum necrotisch te worden (fig. 3B). Opvallend is vooral de grauwgrijze, meestal inwendige, pleksgewijze verkleuring in stelen en grovere nervatuur der geïnoculeerde, primaire bladeren (fig. 3B) en tevens in het basale gedeelte der stengel. Deze verkleuring bleek gevolg te zijn van ophoping van bruine gomachtige stoffen, samengaand met necrose, in parenchymatisch weefsel. Ook zeer typerend is de systemische reactie in de samengestelde bladeren. Deze bestaat uit een plaatselijke, onregelmatig begrensde, tot een algemenere chlorotische

verkleuring van de fijne nerven (fig. 4) en veelal in mindere mate van het aangrenzende bladmoes. Na verloop van tijd kunnen de planten zich min of meer herstellen, waarbij de jongere bladeren een diffuse, grove bontheid vertonen. Bij de klaversoorten bestaan de ziekteverschijnselen uit een min of meer diffuse gevlektheid (fig. 5), die bij de rode klaver soms vrij scherp begrensd kan zijn (fig. 6). Bij witte klaver is de gevlektheid daarentegen vaak zeer onduidelijk, terwijl de symptomen herhaaldelijk geheel ontbreken. Bij komkommer ontstaan op de geïnoculeerde zaadlobben een groot aantal lichtgekleurde puntjes, terwijl de hogere bladeren later diffuus gele vlekken vertonen (fig. 7).

Het bleek, dat het virus zeer besmettelijk is en zelfs door onderling contact van plant op plant kan overgaan. Proeven met warkruid bevestigden de mogelijkheid het virus met de soort *Cuscuta campestris* over te brengen en met dit virus karakteristieke witte-klavermozaïeksymptomen te doen ontstaan in erwten en bonen. Ook bleek het mogelijk, door sap van de voor virusoverdracht benutte warkruidplanten als inoculum te gebruiken, virus hierin aan te tonen.

Proeven om het virus over te brengen met behulp van de erwtebladluis (*Acyrthosiphon pisum* HARRIS), de perzikbladluis (*Myzus persicae* SULZ.) en de zwarte boneluis (*Aphis fabae* SCOP.) na een voorafgaande hongerperiode van één tot drie uur, bij korte zuigtijden op de infectiebron (in een eerste proef variërend van 5–30 min. en in een tweede proef van 7–70 sec.), hadden slechts negatief resultaat. Waarschijnlijk kan het virus niet door bladluizen worden overgedragen.

De resultaten van een aantal proeven ter bepaling der fysische eigenschappen liepen, zelfs voor elke virusisolatie, sterk uiteen. Het thermale inactiveringspunt varieerde van 58°–60° C tot 65–70° C. In één geval kon geen virusactiviteit meer worden aangetoond bij een verdunning van 10^{-4}, terwijl éénmaal met twee isolaties (WKV en TRV) bij een verdunning van 10^{-9} nog infecties konden worden teweeggebracht. Deze laatste verdunningsgrens is voor een plantevirus uitzonderlijk hoog. Het uiteenlopen van de verkregen gegevens vertoont overeenstemming met de grote onderlinge verschillen tussen in de literatuur vermelde waarden (tabel 2). Het virus bleek na 99 dagen bewaring *in vitro* nog een hoge infectiositeit te bezitten. In aan de lucht gedroogde bladeren was 13 dagen na het oogsten nog actief virus aanwezig, in één geval zelfs na 21 dagen, echter niet meer na 29 dagen.

De met de elektronenmicroscoop bestudeerde, draadvormige, ongeveer 475 mμ lange deeltjes komen overeen met die, welke voor het eerst door VAN DER WANT (1954) zijn afgebeeld. Ze komen meestal voor in grote hoeveelheden (fig. 8), zelfs in witte-klaverplanten zonder symptomen.

Eén der door VAN DER WANT (1954) bereide antisera (1.4.15) tegen de isolatie WKV en een nieuw antiserum (7.5.40) tegen de isolatie DEL reageerden positief en op gelijke wijze met alle vier isolaties van het virus (tabel 3).

Uit het onderzoek is gebleken, dat het bestudeerde virus identiek is met het in Amerika optredende „white clover mosaic virus" (ZAUMEYER & WADE, 1935; PIERCE, 1935) en met het in Duitsland voorkomende „Weisskleevirus" (QUANTZ, 1956). De conclusie van JOHNSON (1942), dat het „white clover mosaic virus" van complexe aard zou zijn, kon niet worden bevestigd. Het in Nederland optredende virus komt zowel overeen met het „pea wilt virus" als met het „pea mottle virus", daar *Vigna sinensis* en komkommer beide door de hier beschreven isolaties van het witte-klavermozaïekvirus kunnen worden aangetast. Dit

bleek door middel van teruginoculatie op erwten, bonen, *Vigna* en komkommer. Ook elektronenmicroscopisch was geen verschil waarneembaar in virusdeeltjes na passage door *Vigna* en door komkommer.

De hoge besmettelijkheid van het virus, de overgang van het virus zelfs door contact, het hoge verdunningseindpunt, de lange houdbaarheid *in vitro* en in beperktere mate in gedroogd blad, doen vermoeden, dat het virus veelvuldiger voorkomt dan thans bekend is. Dat het virus nauwelijks is waargenomen op éénjarige gewassen kan een gevolg zijn van het ontbreken van overdragende insekten. Ook is het mogelijk, dat b.v. in bonen de symptomen niet als die van witte-klavermozaïekvirus worden herkend, daar enige weken na de infectie de verschijnselen in de hoger geplaatste bladeren enige overeenkomst vertonen met die van het scherpmozaïek. Mogelijk treedt het virus veelvuldig op in weilanden, gazons e.d. waar het, gezien de hoge besmettelijkheid, waarschijnlijk gemakkelijk kan worden verspreid met het maaien of misschien zelfs door weidend vee.

In vele opzichten vertoont het virus overeenkomst met het X-virus van de aardappel, hoewel er enkele fundamentele verschillen, zoals in serologische eigenschappen en in lengte der deeltjes, bestaan.

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